Methylene Blue Reduced Brain Edema, Neuroinflammation, and Behavioral Deficits associated with a Moderate Traumatic Brain Injury

Honors Research Thesis

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by

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Abstract

Treatment for traumatic brain injury (TBI) is aimed at mitigating the inflammatory response and subsequent complications present immediately after injury as well as the chronic behavioral complications that can persist months or years following the initial injury. Currently, there are no effective treatments to prevent the acute and prolonged neuroinflammation and behavioral deficits after a TBI. Methylene blue (MB) is an anti-inflammatory agent and inducible nitric oxide synthase (iNOS) inhibitor used clinically to reduce inflammation associated with septic shock and hypoperfusion. The purpose of this study was to determine the extent to which MB reduced inflammation and behavioral deficits associated with TBI. Adult (2-3 mo) mice received a sham injury or moderate and diffuse TBI using a midline fluid percussion injury model. Between 5 and 15 min after injury, mice were injected intravenously with sterile water or MB. Mice were either sacrificed 24 h later for tissue collection or examined for anxiety, motor coordination, and depressive-like behavior at one week after injury. Here we show that a moderate TBI increased brain edema in mice 24 h after injury, but was reduced by MB. In addition, 24 h after TBI mRNA expression of several neuroinflammatory mediators including IL-1β and CD14 were increased, but these were also reduced with MB treatment. Moreover, MB decreased the inflammatory profile of trafficking macrophages associated with the brain after injury. Behaviorally, mice with a TBI had impaired motor coordination up to one week after injury and showed evidence of depressive-like behavior at the one week time-point. Treatment with MB, however, improved motor coordination up to baseline and reduced TBI-associated depressive-like behavior. Taken together, these data indicate that MB may serve as an effective clinical treatment to reduce immediate neuroinflammatory events and abrogate chronic behavioral complications associated with TBI.

1. Introduction

1.1 Epidemiological Context

Traumatic brain injury (TBI) is a complex medical condition characterized by acute and long-term structural and functional changes to the brain. TBIs are estimated to account for 1.7 million emergency department visits, 275,000 hospitalizations, and 52,000 deaths per year (Faul, *et al.* 2010). Excluding military related injuries, falls account for largest incidence of TBI cases (35.2%), followed by motor vehicle accidents (17.3%), direct physical impact (16.5%), assault (10%), and unknown/other causes (21%). Adults aged 75 years and older account for the highest rates of TBI-associated hospitalizations. In contrast, children (age 0-4) and teenagers (age 15-19) are the highest risk populations of sustaining a TBI. As a consequence of lifelong TBI management and medical treatment, an aging population, and increased incidence of TBI among military personnel participating in operations in Iraq and Afghanistan, TBIs are expected to account for hundreds of billions of dollars in direct medical costs and estimated loss of productivity over the next decade (Faul, *et. al* 2010). The financial burden of TBI, lack of effective treatment, and mounting epidemiological evidence suggesting a connection between brain injury and neurological conditions including chronic traumatic encephalopathy and major depressive disorder, highlight TBI as a major public health concern.

1.2 Pathophysiology of Traumatic Brain Injury

Traumatic brain injury (TBI) is characterized by two injury phases, an immediate primary injury and chronic secondary injury. The primary injury consists of structural damage directly resulting from biomechanical impact to the brain including diffuse axonal injury, contralateral injury, tissue loss or neuronal death, disruption of neuronal and glial cell membranes, damage to vascular structures including the blood-brain barrier, and other complications associated with skull fractures in open head injuries (Protheroe and Gwinnutt 2011). The extent of these injuries can be inferred through physical examination, use of the well-established Glasgow Coma Scale,

or more directly through neuroimaging, *e.g.* computed tomography (CT) scan to observe cistern formation, parenchymal lesion density, or midline shifts (Farshchian et al. 2012; Greenburg 2010). Observation of these phenomena is highly dependent on the type and severity of injury. All other symptomology of acute clinical relevance including cerebral edema, epi- and subdural hematoma, and ischemia are, for the purposes of this discussion, considered components of secondary injury in accordance with the most recent literature.

The secondary injury is characterized by the pathophysiological responses and immunopathology associated with the release of prostaglandins, free radicals, complement factors, and pro-inflammatory mediators (Ziebell and Morganti-Kossmann 2010). Differential expression of these pathological mediators indicates the involvement of many biochemical pathways that may contribute to and serve as markers of secondary injury severity. Of these inflammatory mediators, interleukin (IL)-1, IL-6, IL-8, IL-10, granulocyte colony-stimulating factor (GC-SF), tumor necrosis factor-α (TNF-α), Fas ligand, and monocyte chemoattractant protein-1 (MCP-1) have received considerable attention over the last decade due to their involvement in specific TBI-associated pathologies or reparative processes (Ziebell and Morganti-Kossmann 2010). For example, IL-1 family cytokines have been shown to exacerbate acute neuronal injury in rodent TBI (Rothwell 1999).

More recent evidence also suggests that these acute inflammatory changes in TBI patients may contribute to chronic neurodegeneration, resulting in reduced white matter density and corpus callosum thickness following a single traumatic brain injury (Johnson, Stewart et al. 2013). Although the molecular mechanisms involved in secondary injury pathogenesis are not well understood, the potential role of inflammation in promoting primary injury-like progressive neurodegeneration highlights the relevance of investigating inflammatory mediators as potential therapeutic targets for long-term TBI management. Moreover, the importance of peripherally derived inflammatory mediators and systemic pathophysiological responses contributing to secondary injury should also be considered. Studies have implicated specific processes such as

platelet serotonin release in serum (Valiyaveettil, Alamneh et al. 2013) and more general changes such as systemic and peripheral inflammatory responses mediated by myeloid-derived cells (Smrcka, Mrlian et al. 2007) following TBI. The relationship between central and systemic inflammation or the extent to which either of these processes contribute to long-term complications in the context of TBI has yet to be characterized.

Other soluble mediators of secondary injury include reactive oxygen species (ROS) and reactive nitrogen species (RNS) that contribute to central and peripheral inflammatory responses and pathophysiological changes in vascular function and blood-brain barrier (BBB) permeability. In a different but comparable model of TBI, recent research has elucidated the role of ROS and RNS in mediating acute cerebrovascular damage and BBB disruption (Abdul-Muneer, Schuetz et al. 2013). Leakiness of the BBB may facilitate peripheral cell infiltration and soluble immune factor entry into the brain. Exposure of these additional inflammatory elements to the brain is hypothesized to underlie the development of various neurodegenerative conditions including Alzheimer's disease and multiple sclerosis (de Vries, Kooij et al. 2012). Further understanding of the role these mediators play in pathophysiology and their potential utilization as biomarkers of injury severity in human TBI is of widespread interest in current neurotrauma research.

Neuroinflammation, ROS and RNS activity, and cerebrovascular damage not only contribute to exacerbated tissue loss and neuronal death beyond that of the primary injury, but have also been implicated in the development of depression in experimental models (Dantzer, O'Connor et al. 2008) and epidemiological studies. In one large scale study (n=722) of human TBI in 2001, 42% of injured patients were determined to have comorbid major depressive disorder (MDD), based on DSM-IV criteria, an average of 2.5 years post injury (Kreutzer, Seel et al. 2001). In a separate study published the same year (n=91), the authors found that 33% of TBI patients were also diagnosed with MDD. Comorbid cases of TBI and MDD have received considerable attention in the media following the high profile suicides of several professional

players in the National Football League and struggles of combat veterans of Iraq and Afghanistan who suffered head trauma. Although TBI is not necessarily directly linked to suicide, the comorbid psychopathologies associated with injury, including MDD, substance use disorder, assaultive behavior, engagement in dangerous activities, and self-directed violence among many others have all been identified as significant risk factors for suicide (Olson-Madden, Forster et al. 2012). Other long-term complications associated with TBI include post-concussion syndrome (PCS) and chronic traumatic encephalopathy (CTE). Description, diagnosis, and treatment of PCS and CTE remain controversial due to the ambiguous etiology, non-specificity of clinical symptoms, and limitations of current studies. PCS is broadly characterized by a variety of somatic *e.g.* headache, vertigo and psychological *e.g.* irritability, anxiety symptoms often following a single TBI (Leddy, Sandhu et al. 2012). In comparison, CTE is broadly characterized by similar somatic and psychological symptoms, but also several unique post-mortem anatomical markers, *e.g.* neurofibrillary tangle formation, amyloid plaques often observed following repetitive, mild TBIs (Turner, Lucke-Wold et al. 2012; Greenburg 2010). Evaluation and treatment of PCS and CTE remain an ongoing problem for clinicians. Currently, there are no universally accepted or effective medicinal, surgical, or therapeutic strategies to manage acute and chronic TBI complications.

1.3 Neuroimmune Basis of Secondary Injury Severity and Behavioral Deficits

Recent research by our laboratory has emphasized the role of microglia, the innate immune cell of the central nervous system (CNS), in mediating acute inflammatory processes in the brain when activated in relation to aging, lipopolysaccharide (LPS) insult, repeated social defeat, early life infection, and TBI. Preliminary data from our lab demonstrate that following a moderate and diffuse TBI in mice, microglia become primed (*i.e*., increased MHCII expression). This is relevant because previous research has established that primed, $MHCII^{+}$ microglia are responsible for exaggerated neuroinflammation. In particular, aging alone was found to promote

a primed, $MHCII^+$ microglial population in the brain (Godbout, Chen et al. 2005). Following an inflammatory challenge, these $MHCII^+$ microglia become hyper-active and secrete elevated levels of the pro-inflammatory cytokine IL-1β into the brain (Henry, Huang et al. 2009) resulting in a prolonged sickness response (Godbout, Chen et al. 2005) and induction of depressive-like behavior (Godbout, Moreau et al. 2008). Indeed, our previous TBI data indicate that one month following a TBI, microglia had increased MHCII expression and, following a peripheral inflammatory challenge with LPS, these microglia became hyperactive resulting in exaggerated pro-inflammatory cytokine production (*i.e.,* IL-1β, iNOS, TNFα). Importantly, this exaggerated neuroinflammation resulted in prolonged sickness behavior in TBI mice injected with LPS. Previous studies have also shown that TBI mice demonstrated exaggerated depressive-like behavior one week and one month post-injury.

Upregulation of pro-inflammatory cytokines due to LPS immune challenge is coupled with microglial activation. Microglial activation is determined through ionized calcium-binding adapter molecule 1 (Iba1) immunohistochemical staining in which cells demonstrate an activated morphology characterized by shortened and thickened cell processes and enlarged cell bodies (Corona, Huang et al. 2010). An increase in the activated microglia phenotype can be observed in the CA1 region of the hippocampus one month following TBI. Exaggerated activation of microglia coupled with the significant upregulation of pro-inflammatory cytokines has been linked with depressive-like behavior in mice (Godbout, Moreau et al. 2008). Increased immobility time in tail suspension test (TST) (Corona, Huang et al. 2010) and forced swim test (FST; Godbout, Moreau et al. 2008) are indications of this depressive-like behavior. We hypothesized that acute inflammatory processes mediated by activated microglia in the brain following injury, underlie secondary injury pathophysiology, and facilitate the development of chronic behavioral deficits associated with TBI.

1.4 Recent Therapeutic Strategies

Conventional TBI treatment in an acute care setting is focused on symptomatic management of life-threatening complications directly or indirectly related to head trauma *e.g.* spinal cord or musculoskeletal injury. These complications include hematoma formation, detrimental changes in cerebral blood flow or cerebral perfusion pressure, threatening increases in intracranial pressure, and malignant cerebral edema (Greenburg 2010). Following acute TBI management, therapeutic strategies addressing the appearance of PCS or other behavioral and cognitive deficits is highly variable. A recent review summarized the multitude of treatment paradigms available to clinicians in order to address PCS symptoms. These included withdrawal from physical or cognitive exertion immediately following injury, psychotherapy, antidepressant use, sensorimotor rehabilitation, and ultimately a progressive increase in cognitive and physical activity approximately one week following injury (Leddy, Sandhu et al. 2012).

No widely adopted medicinal strategies have, to date, been shown to ameliorate neuroinflammation, cerebrovascular damage, and behavioral deficits associated with human TBI. However, several medicinal therapies have gained considerable support and have been successful. Use of a chemical formulation of progesterone, termed BHR-100, recently entered a Phase 3 clinical trial, SyNAPSe®, in 2010 to evaluate its effectiveness in treating severe TBI.

Regardless of the success of the SyNAPSe® clinical trial, medicinal treatment of TBI remains an area of intense investigation. Here we provide support for the utilization of methylene blue, a potential therapeutic drug, for the treatment of neuroinflammation, cerebrovascular damage, and behavioral deficits associated with TBI. Methylene blue (MB) is unique in its broad range of molecular, cellular, and systemic targets in the central nervous system. Of particular relevance to TBI, MB is a potent constitutive and indirect nitric oxide synthase (iNOS) inhibitor that targets both the receptor for nitric oxide, soluble guanylate cyclase (sGC), and iNOS directly. In this regard, the effect of MB on the iNOS/sGC biochemical pathway is well-established. Furthermore, the redox activities of MB facilitate the oxidation of both ROS and RNS, and highlight its relevance as a powerful antioxidant (Oz, Lorke et al.

2011). In the context of TBI, the antioxidant and iNOS inhibitory activities of MB may serve to alleviate the oxidative and nitrosative stresses following injury that mediate BBB disruption and cerebrovascular damage. Recent evidence has also shown the beneficial effects of sGC inhibition using zinc protoporphyrin IX on edema formation, BBB stability, and serotonin levels in a comparable model of closed TBI (Vannemreddy, Ray et al. 2006). Moreover, there are several clinical cases in the literature reporting successful MB therapy in severe cases of refractory septic shock through restoration of hemodynamic stability (Juffermans, Vervloet et al. 2010; Dumbarton, Minor et al. 2011). Recent evidence also suggests MB has potent microglia specific anti-inflammatory activities on IL-6 and TNF-α formation in a murine model of amyotrophic lateral sclerosis. Taken together, these findings implicate the role of MB in attenuating secondary injury pathology associated with microglia specific neuroinflammation and ROS/RNS dependent cerebrovascular damage (Dibaj, Zschuntzsch et al. 2012).

Alternatively, MB mimics the anti-depressive functions of selective serotonin reuptake inhibitors and monoamine oxidase inhibitors, in addition to modulation of both dopaminergic and glutamatergic neurotransmitter systems to produce chlorpromazine-like antipsychotic effects (Oz, Lorke et al. 2011). Potential reversal of functional deficits in behavior and cognition following TBI is of considerable clinical importance due to the inefficacy of current management strategies addressing comorbid depression. For these reasons and the other anti-inflammatory and anti-oxidant activities discussed previously, we hypothesized that MB treatment following TBI would attenuate microglia mediated neuroinflammation, cerebrovascular damage, and behavioral complications (Fig. 1).

2. Materials and Methods

2.1 Animal Use Protocols

Adult (2-3 mo) BALB/C mice were housed in polypropylene cages and maintained at 25 °C under a 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. All

proposed procedures are in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were experimental conditions previously approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Three separate cohorts of mice were used for this study. In the first experiment, blood and brain tissue was extracted for qPCR and flow cytometric analysis 24 h post-TBI. In the second experiment, brain tissue was extracted for measurement of edema in the cortex 24 h post-TBI. In the third experiment, mice were examined for anxiety, motor coordination, and depressive-like behavior up to one week following injury and were euthanized 1 m post-TBI for a separate study (data not shown).

2.2 Fluid Percussion Injury (FPI)

Mice received a craniotomy 3 mm in diameter mid-way between Bregma and Lambda under ketamine (100 mg/kg body weight [BW]) and xylazine (10 mg/kg BW) anesthesia. Following recovery from anesthesia (3-4 h), mice received a midline and diffuse, moderate TBI using the Fluid Percussion Injury (FPI) Device (Custom Design&Fabrication, Virginia Commonwealth University, Richmond, VA) which induced injury with a micro fluid-pulse of saline onto the brain through the craniotomy (Lifshitz, Witgen et al. 2007). Sham control mice were subjected to all experimental conditions, excluding the injury. The FPI model induces a consistent, moderate and diffuse TBI with a 10-20% mortality rate (Lifshitz, Witgen et al. 2007; Lifshitz 2008).

2.3 Intravenous (i.v.) Methylene Blue Injection

Mice received a 100 µl *i.v.* injection of vehicle (ddH_2O) or dilute 1% methylene blue solution (Akorn, Inc; 2 mg/kg BW) between 5 and 15 min following injury. No relevant side effects have been identified in our model or clinically at this dosage.

2.4 Blood and Brain Extraction

In the first study, mice were euthanized using a lethal dose of ketamine (200 mg/kg BW) and xylazine (20 mg/kg BW) 24 h following injury for tissue collection. Blood was extracted via cardiac puncture for flow cytometric analysis of plasma. Brains were extracted and a 1 mm coronal brain slice through the FPI impact site for gross mRNA analysis was removed. The remaining tissue was used for microglia isolation.

2.5 Microglia Isolation

Brain tissue was homogenized 24 h post-injury. Enriched microglia were isolated using a Percoll density gradient extraction protocol as previously described (Henry, Huang et al. 2008). We have previously determined that this results in an “enriched” population of 85-90% pure microglia. Enriched microglia were then used for mRNA and flow cytometric analysis (Henry, Huang et al. 2009; Wynne, Henry et al. 2010).

2.6 Quantitative PCR (qPCR) Analysis of Brain Slice and Microglia

mRNA from the 1 mm coronal brain slice and microglia was isolated using a Tri-Reagent RNA Isolation Protocol or spin column PrepEase RNA Spin Kit (Affymetrix) respectively. Isolated mRNA was converted to cDNA and analyzed using qPCR to determine changes in pro- (IL-1β, CD14, iNOS) and anti-inflammatory (IL-4Rα, IL-4, IL-10, and arginase I) mediator expression following TBI and MB administration as previously described (Fenn, Henry et al. 2012).

2.7 Flow Cytometric Analysis of Plasma and Microglia

Analysis of plasma and microglial protein surface expression by flow cytometry was performed as previously described (Henry, Huang et al. 2008; Henry, Huang et al. 2009). In brief, Fc receptors were blocked. Plasma was incubated with anti CD11b and anti GR-1 to

characterize monocyte populations in circulation. Microglia were incubated with anti CD11b, anti CD14, anti CD45, or anti Ly6C antibodies respectively to characterize inflammatory microglia and trafficking macrophage phenotypes (eBioscience, CA). Antigen expression was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Ten thousand events were recorded. For each antibody, gating was determined based on appropriate negative isotype stained controls. Flow data were analyzed using FlowJo software (Tree Star, CA).

2.8 Measurement of Edema

In the second experiment, mice were euthanized using a lethal dose of ketamine (200 mg/kg BW) and xylazine (20 mg/kg BW) 24 h following injury. The brain was extracted, and the left and right cortices were dissected out and immediately weighed. Cortices were oven-dried at 72 °C for 24 h and weighed again to calculate relative percent water loss. No differences in percent water loss were observed when brain tissue was oven-dried at 72 °C for 48 or 72 h.

2.9 Behavioral Tests

In the third experiment, mice were examined for deficits in anxiety, motor coordination, and depressive-like behavior up to one week following injury. Mice were euthanized 1 m following injury for a separate analysis (data not shown).

The anxiety test was determined as previously described (Kinsey, Bailey et al. 2007; Wohleb, Hanke et al. 2011; Wohleb, Fenn et al. 2012). In brief, experimental mice were placed in the test apparatus that consisted of a 40 cm *x* 40 cm *x* 25 cm Plexiglas box in dim light. Mice were placed into the corner of the open-field and locomotor activity was recorded for 5 min using an automated system and analyzed using VersaMap software (AccuScan Instruments, Columbus, OH). Schematic diagrams of mouse position and the number of center entries, into the open field, were recorded for analysis.

Motor coordination was determined using a Rotarod testing apparatus before and after injury. Mice were placed on the stationary rod 3 d before injury in order to be acclimated to the Rotarod testing setup. Mice were placed on the rod at constant revolution (5 revolutions/min) 2 d before injury to complete acclimation to the testing paradigm. Motor coordination was then determined by the time spent on the Rotarod at the experimental angular velocity (10 revolutions/min) and acceleration (0.2 revolutions/min^2) 1 d before injury. Testing continued at 4 h and 1, 3, 5, and 7 d post-TBI. Each mouse was tested in three separate trials. The first and second trials were performed consecutively; the third trial was performed 10 min after the second trial. The two closest performance times among the were averaged and used for each mouse in this analysis.

The tail suspension test (TST) was used to determine depressive-like behavior. This model is an established and accepted model of resignation behavior in mice (Godbout, Moreau et al. 2008; Corona, Huang et al. 2010). In brief, mice were suspended by their tail in a 32 *x* 33 *x* 33 box under dim conditions for 10 min. Immobility times, as a measure of resignation behavior, were recorded by a trained observer blind to experimental conditions.

2.10 Statistics

To ensure a normal distribution, data was subjected to Shapiro-Wilk test using Statistical Analysis Systems (SAS) software (Cary, NC). Observations greater than 3 interquartile ranges from the first and third quartile were considered outliers and were excluded in the subsequent analysis. To determine significant main effects and interactions between main factors, data was analyzed using one- (TBI; MB) or two- (TBI *x* MB) way ANOVA using the General Linear Model procedures of SAS. When appropriate, differences between treatment means were evaluated by an F-protected t-test using the Least-Significant Difference procedure of SAS. Significance was determined at $p < 0.05$ and indicated by (*) or letter notation (a, b) to demonstrate statistically significant differences between groups. Where noted, a simple one-tailed T-test with

equal variance was used to determine significance ($p < 0.05$) for *Post-hoc* analysis and was indicated by (+).

3.0 Results

3.1 MB Abrogated TBI-induced Inflammatory Mediators in the Brain

In previous studies using our FPI model of TBI, we found microglial (IL-1β) and astrocytic (GFAP) markers of inflammation were significantly upregulated 4 and 72 h post-TBI respectively in the cortex and hippocampus (data not shown). Our aim was to first examine the effect of MB treatment on pro- and anti-inflammatory markers in the 1 mm coronal brain slice as an estimate of gross mRNA expression in the brain. mRNA concentrations of several inflammatory: A) IL-1β, B) iNOS, and C) CD14 and anti-inflammatory mediators: D) arginase I, E) IL-10, F) IL-4Rα and G) IL-4 were measured 24 h following injury (Fig. 2). Our preliminary results have shown that TBI induced a noted upregulation of IL-1β and CD14, both of which were reduced by MB. Alternatively, MB increased expression of anti-inflammatory mediators in the brain, including IL-4 and IL-10; however, no significant statistical interactions were identified. These data indicate that TBI induced an acute inflammatory response in the brain that was reduced by MB.

3.2 MB Abrogated TBI-Induced Expression of Inflammatory Mediators in Microglia

Once the effect of MB treatment on inflammatory mediators was established in whole brain tissue, we continued our studies on microglia specific populations. mRNA concentrations of several inflammatory: A) IL-1β, B) CD14, C) iNOS and anti-inflammatory mediators: D) arginase I, E) IL-10 and F) IL-4Rα were measured from isolated microglia (Fig. 3). Our aim was to evaluate the role of microglia in mediating inflammatory responses 24 h following injury through analysis of both pro- and anti-inflammatory mediators. Again, both IL-1β and CD14 were upregulated following injury, and were subsequently reduced after treatment with MB.

Furthermore, expression of IL-10 increased with MB treatment. Arginase I was significantly upregulated following injury (TBI *x* Vehicle) compared to Sham *x* Vehicle and TBI *x* MB (p = < 0.03) and to a lesser extent Sham *x* MB (p = 0.10). No other significant interactions were identified. These data indicate that microglia significantly upregulated arginase activity 24 h following injury and contributed to acute neuroinflammation. The effect of MB on arginase indicated that MB had a significant effect on microglia; however, the mechanism of this activity remains to be elucidated.

3.3 MB Did Not Reduced CD14 Protein Expression on Microglia after TBI

Although qPCR analysis provided a broad profile of pro- and anti-inflammatory mediator expression in the whole brain and microglia, the physiological relevance of these data was unsubstantiated. We chose to examine the relative percentage of $CD11b^{+}/CD14^{high}$ microglia, an established reactive and inflammatory phenotype, through flow cytometric analysis of surface protein expression 24 h following injury (Fig. 4). CD14 expression significantly increased with TBI (p = 0.0004) but there was no significant reduction following MB treatment (p = 0.6). These data indicate that microglial activation, as evaluated through CD14, associated with TBI but was unaffected by MB treatment.

3.4 MB Had No Effect on Trafficking Macrophages after TBI

The microglia extraction protocol used in this experiment (Materials and Methods 2.5) cannot differentiate between isolation of microglia and trafficking macrophages. Macrophage populations in whole brain tissue can however be identified through flow cytometric analysis of macrophage specific surface protein expression ($CD11b^{+}/CD45^{high}$ or $CD11b^{+}/Ly6C^{high}$). Analysis of $CD11b^{+}/CD45^{high}$ cells was conducted to measure the relative percentage of macrophages associated with the extracted brain sample 24 h following injury (Fig. 5A). The

relative percentage of $CD11b^{+}/CD45^{high}$ cells significantly increased with TBI (p = 0.015) but there was no significant reduction with MB treatment (p = 0.65).

Moreover, we attempted to examine differences in the relative percentage of inflammatory trafficking macrophages, $CD11b^{+}/Ly6C^{high}$, associated with the brain 24 h post-injury (Fig. 5B). No significant differences were attributed to TBI (p = 0.18) or MB (p = 0.66); however, there was a minor interaction between the Sham *x* Vehicle and TBI *x* Vehicle groups (p = 0.08) that was also observed through *Post-hoc* analysis (p = 0.06). Taken together, these data indicate that MB has no effect on TBI-induced macrophage trafficking, but the inflammatory profile of these cells may be attenuated by MB.

3.5 MB Attenuated the Inflammatory Profile of Myeloid Cells in Circulation

Analysis of trafficking macrophages was extended to myeloid-derived monocyte populations in circulation in order to determine the phenotype of prospective inflammatory cells 24 h following injury. The relative percentage of $CD11b^{+}/GR\text{-}1^{+}$ cells through flow cytometry (quantitative analysis and representative dot plots shown in Fig. 6A and Fig. 6B respectively) were measured. No significant interactions were identified; however, there was a minor interaction associated with TBI (p = 0.07) that was reversed by MB (p = 0.14). These data indicate that TBI can affect the inflammatory profile of peripheral cells, and that this interaction may be reduced by MB treatment. The extent to which these activated monocyte populations affected macrophage trafficking warrants further investigation.

3.6 MB Reduced TBI-induced Edema

Although not thoroughly characterized, our previous studies indicated that edema was induced by our TBI model and could be successfully measured (data not shown). Considering the clinical relevance of edema in human TBI, our aim was to reduce the formation of edema with MB treatment. Using the same methods as our previous experiments, the left and right

cortices were removed from each extracted brain 24 following injury and oven-dried to evaluate the relative percent water loss as a measure of edema (Fig. 7). No significant interactions were identified; however, the TBI *x* Vehicle group differed from the Sham *x* Vehicle group by a simple, one-tailed T-Test (p = 0.10), indicating that edema was successfully induced by our model and that MB treatment may have a reversing effect. No other minor interactions by a simple, one-tailed T-test were observed between the remaining groups.

3.7 MB Reversed TBI-induced Deficits in Anxiety, Motor Coordination, and Depressive-like Behaviors

Our lab has extensively studied the effect of inflammatory mediators in the brain and periphery on behavior. Here we have used the same behavioral tests in the context of TBI and MB treatment. Our aim was to connect the inflammatory profile of microglia to observable behavior deficits up to 7 d following injury. Mice were tested for anxiety behavior (representative and quantitative analysis of the test in Fig. 8A and Fig. 8B respectively) in an open field setup (Materials and Methods 2.9). No significant interactions were identified, but there was a noted increase in the number of center entries induced by TBI compared to sham controls that was reduced by MB (Fig. 8B).

Furthermore, we used a Rotarod test to evaluate the effect of TBI on motor coordination (Fig. 8C). Time spent on the Rotarod was recorded up to 7 d following injury. Again, no significant interactions were identified but there was an observable separation among the treatment groups at 7 d with the TBI *x* Vehicle group performing at baseline, whereas the Sham *x* Vehicle, Sham *x* MB, and TBI *x* MB groups had improved above baseline performance.

Lastly, mice were also examined for depressive-like behavior using our tail suspension test (TST; Materials and Methods 2.9). Immobility times were recorded as a measure of depressive-like activity (Fig. 8D). No significant interactions were identified, but there was an increase in immobility time associated with TBI that was reversed by MB treatment. Overall,

these data indicate that MB promoted self-preservation behavior in the open field test, reversed motor coordination benefits in the Rotarod test, and attenuated depressive-like behavior in the TST associated with TBI up to 7 d following injury.

4. Discussion

In our previous studies, inflammatory microglial and astrocytic markers were differentially upregulated in relation to time and brain region following injury (data not shown). An important finding of this preliminary study was to demonstrate the development of an acute inflammatory response in the whole brain (Fig. 2) and microglia (Fig. 3) 24 h after TBI that was reduced by MB treatment. In particular, the inflammatory mediators IL-1β (Fig. 2A and Fig. 3A) and CD14 (Fig. 2B and Fig. 3C) were upregulated in the 1 mm coronal brain section and microglia after injury. Expression of these markers was downregulated with MB. Alternatively, expression of the anti-inflammatory cytokine IL-10 (Fig. 2E and Fig. 3E) increased with MB treatment following injury in the brain slice and microglia. Taken together, these data indicate that acute neuroinflammation associated with TBI was reduced by MB treatment.

Furthermore, microglial arginase I was significantly upregulated with TBI (Fig. 3D) but reduced with MB (p = < 0.03), whereas no significant interaction was identified with iNOS activity. The clinical relevance of these data is unclear. Recent evidence has shown that arginase I and iNOS are inversely regulated by SHP-1, a tyrosine phosphatase in microglia, in response to innate immune system activation (Bonaparte, Hudson et al. 2006). Moreover, the pathophysiological interaction between arginase and iNOS are unclear. For example, arginase overexpression is associated with the consumption of bioavailable L-arginine that can hasten the iNOS-mediated formation of reactive nitrogen species (RNS; Wink, Hines et al. 2011). As previously discussed, RNS disrupt the blood-brain barrier and damage the cerebrovasculature (Abdul-Muneer, Schuetz et al. 2013). Although our model has not been shown to induce neuronal death (Lifshitz, Kelley et al. 2007), we anticipate the significant upregulation of

arginase I associated with TBI is indicative of cellular repair of damaged neurons and glial cells. In this regard, reduction in arginase I expression following MB treatment may indicate acute neuroprotective activity and limit the necessity of arginase-mediated cellular repair processes. In contrast, the effects of arginase I downregulation on RNS formation and the pathophysiological consequences of increased L-arginine bioavailability remain to be elucidated.

Our data support the idea that microglial activation and acute neuroinflammation persist in the brain 24 h following TBI. In support of this idea, our first aim was to establish the phenotype of resident microglia and trafficking macrophages at the protein level through flow cytometric analysis. The relative percentage of $CD14^{high}$ microglia was significantly upregulated with TBI but no effect was observed following MB treatment. Rather, histological analysis of microglial morphology through Iba1 immunohistochemical staining would provide a more appropriate analysis of microglial activation (Corona, Huang et al. 2010) and the therapeutic effect of MB. Moreover, the relative percentage of $CD11b^{+}/CD45^{high}$ cells significantly increased following TBI ($p = 0.015$) but was unaffected by MB. In contrast, no significant interactions were associated with TBI or MB on the relative percentage of $CD11b^{+}/Ly6C^{high}$ cells. *Post-hoc* analyses of these data indicate that MB treatment may attenuate the inflammatory profile of these cells ($p = 0.06$). Taken together, these data indicate that macrophage trafficking is associated with TBI, and MB treatment may attenuate the inflammatory profile of the cells following injury; however, the specific phenotype and infiltrative capacities of trafficking macrophages after TBI remain uncharacterized in this study. Alternatively, our second aim was to examine the role of prospective infiltrative and inflammatory monocytes through flow cytometric analysis. We determined an increase in the relative percentage of inflammatory $CD11b^{+}/GR\text{-}1^{+}$ cells that had a tendency to associate with TBI ($p = 0.07$) but was reduced with MB treatment ($p = 0.14$). These data indicate that MB attenuated the inflammatory profile of circulating myeloid cells that may further serve to limit the infiltration of trafficking macrophages

in the brain. Overall, these data demonstrate that MB has an effect on resident microglia, trafficking macrophages, and circulating myeloid cells that is worthy of further investigation.

An interesting finding from this study was that edema formation was successfully induced and measured in our FPI model of TBI. Although no major significant interactions were observed, the relative percentage of water loss in the TBI *x* Vehicle group had a tendency to differ significantly from the remaining groups (Sham *x* Vehicle, Sham *x* MB, and TBI *x* MB) by a simple, one-tailed T-test (0.10). These outcomes are encouraging and suggest that MB may serve as an effective medicinal treatment for post-TBI edema formation, particularly in comparison to conventional treatment strategies. In this context, MB has emerged as a prime candidate for potential therapeutic use in reducing brain edema. In addition, due to the surgical protocol required for our FPI model (Materials and Methods 2.2), we anticipate our data reflect a conservative estimate of edema formation in the TBI *x* Vehicle and TBI *x* MB groups *i.e.* all mice underwent craniectomy (3 mm burr hole) without damage to the meningeal layers. We argue that in absence of the craniectomy, the formation of edema and therapeutic efficacy of MB would be enhanced.

These data alone characterizing the anti-inflammatory and medicinal effects of MB treatment on TBI provide a strong foundation for the clinical application of MB in CNS trauma. A novel aspect of this study however was the effect of MB treatment on anxiety, motor coordination, and depressive-like behaviors up to 7 d following injury. In this regard, we have established the role of neuroinflammation in the development of depressive-like behavior (Godbout, Moreau et al. 2008). Clinically, neuroinflammation has also been shown to mediate neurologic complications. For example the chemotherapeutic drug, ifosfamide, is well-known to induce encephalopathy and neurotoxicity associated with behavioral complications such as delirium and depressive psychosis. In these cancer patients, MB is the treatment of choice and has been shown to completely reverse neurologic and behavioral symptoms (Pelgrims, De Vos et al. 2000; Brunello, Basso et al. 2007). Our data contribute to the literature characterizing the

activity of MB on reversing these functional deficits. Likewise, in an open field test for anxiety behavior, there was a noted increase in the number of center entries, an inverse measure of self-preservation and anxiety behavior, associated with TBI that was reversed by MB treatment (Fig. 8B). These data suggest TBI inhibited baseline anxiety behavior in BALB/C mice and MB acted to restore this phenotype. These data are supported by corroborating findings in human TBI *e.g.* engagement in dangerous activities and self-directed violence (*i.e.* inhibition of self-preservation behavior) are characteristic TBI symptoms. Furthermore, in the Rotarod test for motor coordination, there was an observable separation among the experimental groups in performance 7 d after TBI (Fig. 8C). At this time point, the Sham *x* Vehicle, Sham *x* MB, and TBI *x* MB outperformed the TBI *x* Vehicle group. Lastly, in our tail suspension test for depressive-like behavior, there was an increase in immobility time in the TBI *x* Vehicle group that was reduced by MB treatment (Fig. 8D). As previously discussed, the prevalence of major depressive disorder in TBI patients is a well characterized complication in humans. No significant interactions were identified in these preliminary studies; however, our results suggest that a single dose of MB (2 mg/kg) immediately following injury may be sufficient to prevent behavioral complications up to 7 d post-TBI. The efficacy of multiple MB injections, at or higher than the current dosage (2 mg/kg), on acute neuroinflammation or associated behavioral complications remains to be elucidated. These data highlight the clinical relevance of ameliorating TBI-induced anxiety and major depressive disorder in humans through medicinal therapy, and establish MB as a potential drug for these purposes.

In conclusion, our model of moderate fluid percussion-induced traumatic brain injury, successfully demonstrated the development of acute neuroinflammation, edema, and behavioral deficits 24 h following injury. In particular, TBI upregulated the expression of inflammatory IL-1β and CD14 in the whole brain and resident microglia. The specific role of these microglia in mediating acute neuroinflammation, edema, and behavioral deficits associated with TBI remain to be elucidated; however, these data suggest resident microglia mediate IL-1β and arginase

dependent pathophysiological responses. Additional studies will be required in order to examine microglial activation at various time points following injury. Furthermore, TBI increased the number of inflammatory monocytes associated with the brain and in circulation. Lastly, TBI induced acute behavioral deficits in anxiety, motor coordination, and depressive-like behavior up to 7 d post-injury. Intravenous administration of MB immediately following injury acted to reduce inflammatory gene expression in the whole brain and resident microglia, decreased the inflammatory profile of trafficking macrophages and peripheral monocytes 24 h after TBI, and reversed deficits in anxiety, motor coordination, and depressive-like behaviors up to 7 d following injury. Taken together these results indicate that early intervention with MB following moderate injury reduced inflammatory and behavioral consequences of TBI. Thus, MB treatment could reduce immediate life-threatening complications, including malignant cerebral edema. Moreover, early MB treatment has the potential to promote long-lasting neurologic improvements in TBI patients including reduced development of major depressive disorder. Therefore, MB administration after TBI may be a novel therapy for mild to moderate TBI.

FIGURE 1

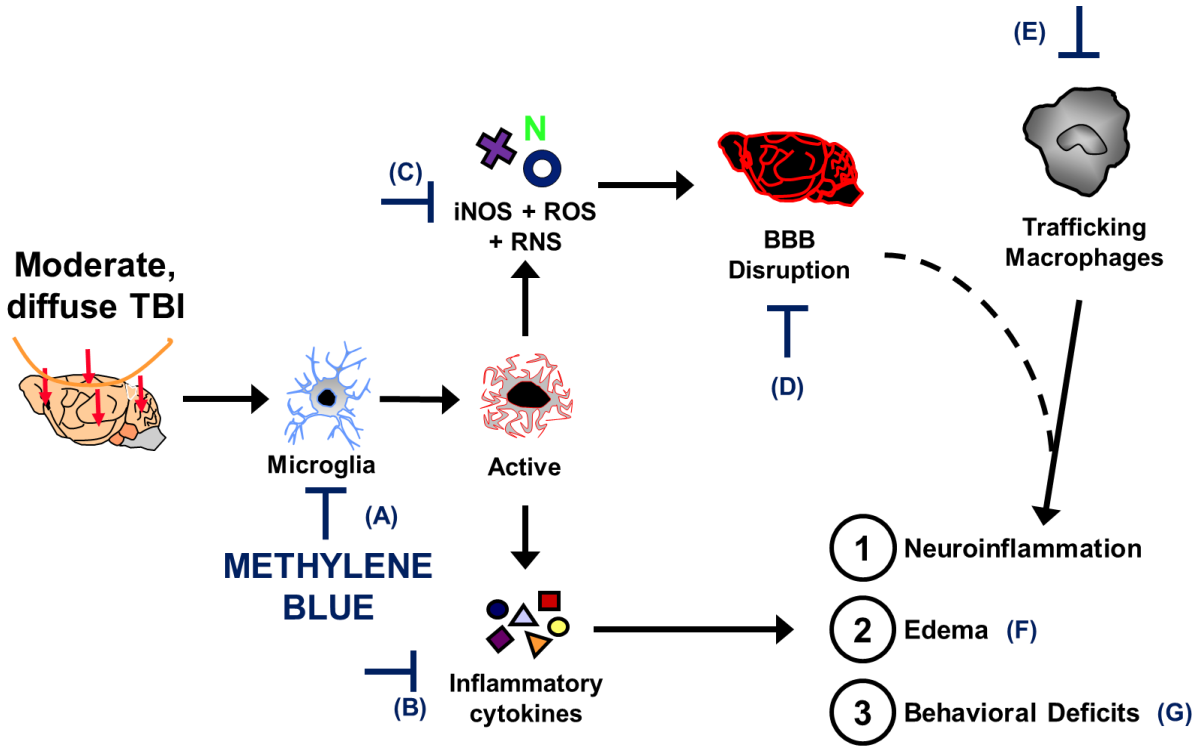
(E)
N
(C)
iNOS + ROS
+ RNS
BBB
Disruption
Trafficking
Macrophages
Moderate,
diffuse TBI
(D)
Microglia
Active
(A)
METHYLENE
BLUE
1 Neuroinflammation
2 Edema (F)
3 Behavioral Deficits (G)
(B)
Inflammatory
cytokines

FIGURE 2

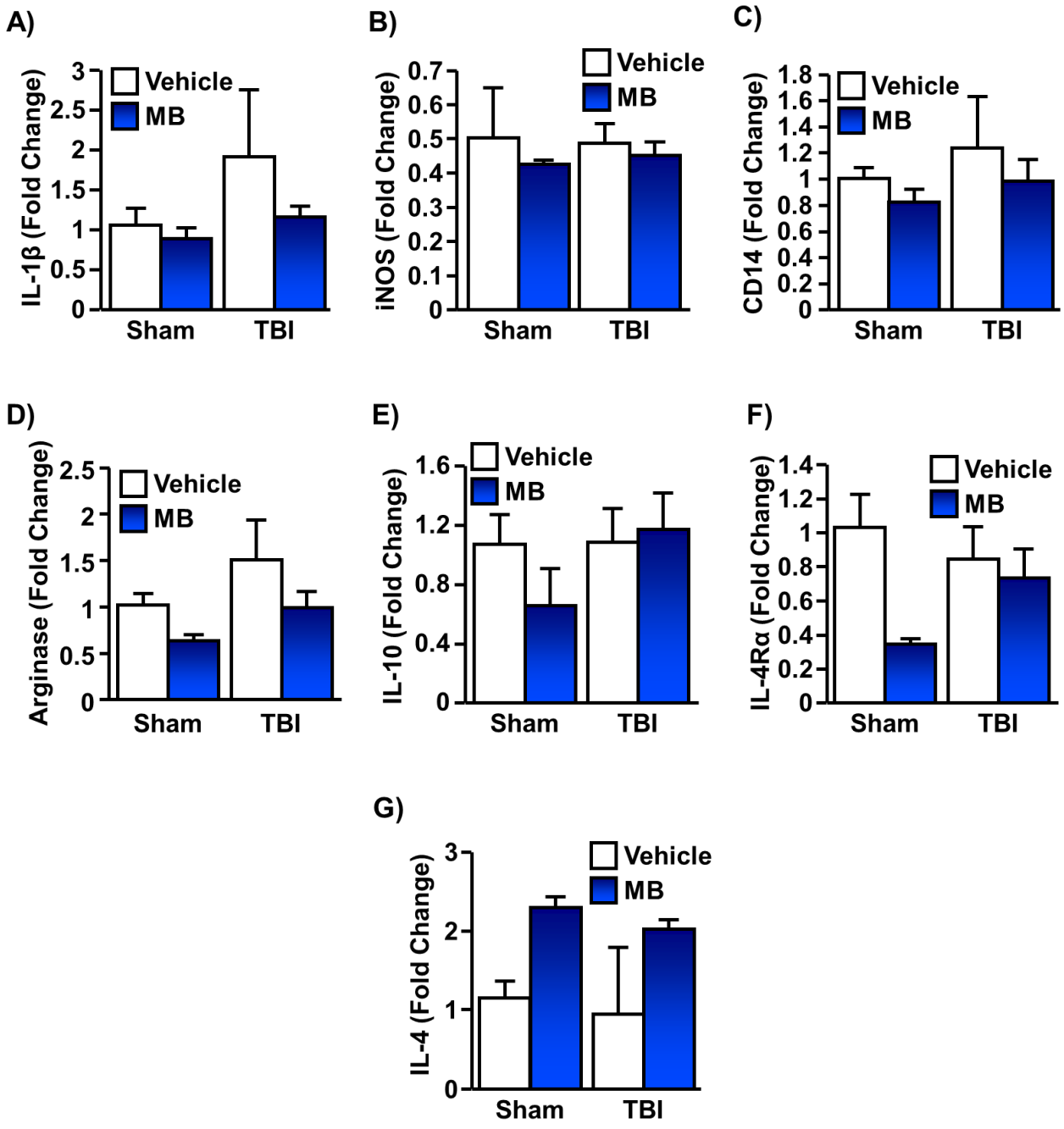
A)
IL-1β (Fold Change)
3
2.5
2
1.5
1
0.5
0
Vehicle
MB
Sham
TBI
B)
iNOS (Fold Change)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Vehicle
MB
Sham
TBI
C)
CD14 (Fold Change)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
MB
Sham
TBI
D)
Arginase (Fold Change)
2.5
2
1.5
1
0.5
0
Vehicle
MB
Sham
TBI
E)
IL-10 (Fold Change)
1.6
1.2
0.8
0.4
0
Vehicle
MB
Sham
TBI
F)
IL-4Rα (Fold Change)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
MB
Sham
TBI
G)
IL-4 (Fold Change)
3
2
1
0
Vehicle
MB
Sham
TBI

FIGURE 3

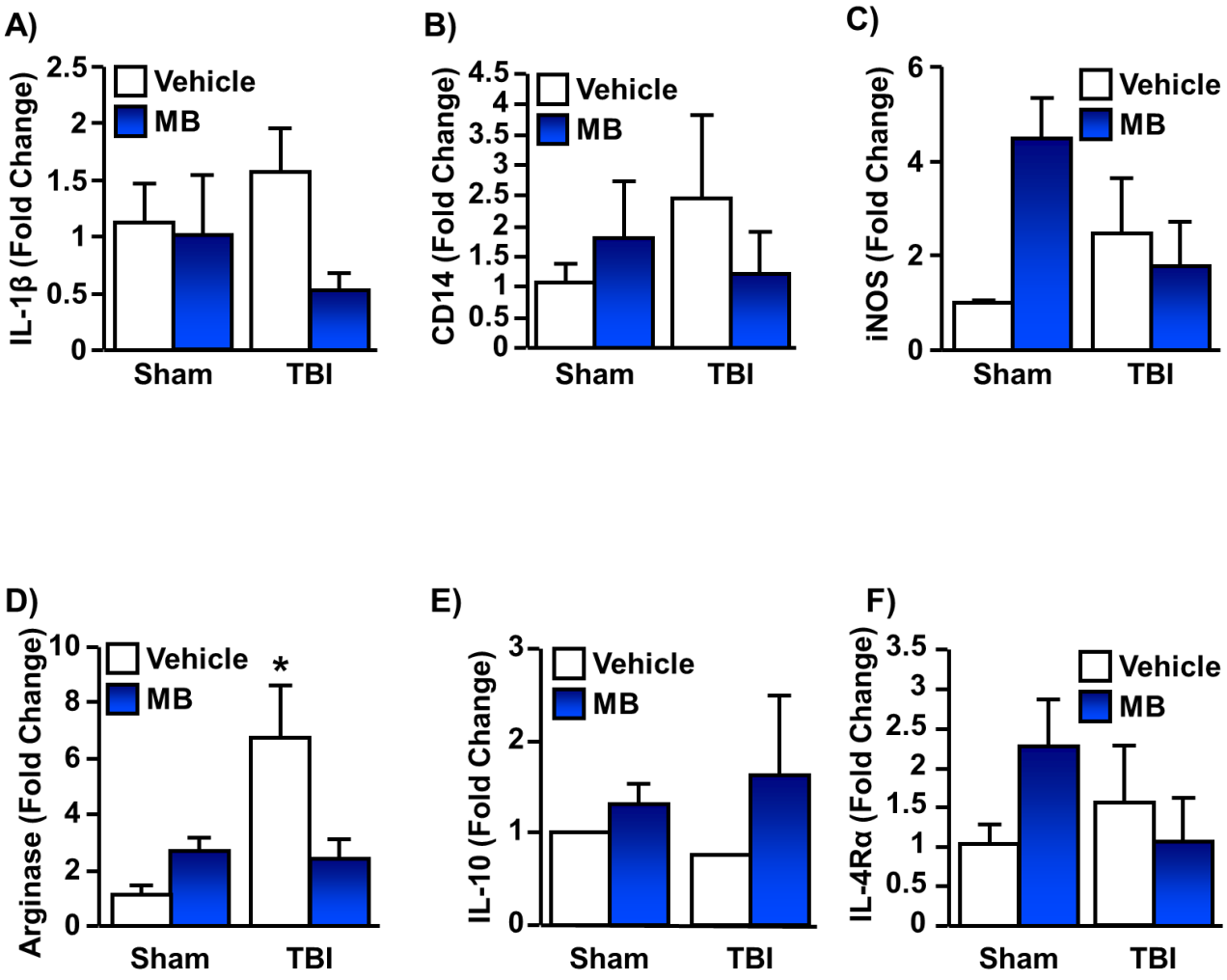
A)
IL-1β (Fold Change)
2.5
2
1.5
1
0.5
0
Vehicle
MB
Sham
TBI
B)
CD14 (Fold Change)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
MB
Sham
TBI
C)
iNOS (Fold Change)
6
4
2
0
Vehicle
MB
Sham
TBI
D)
Arginase (Fold Change)
10
8
6
4
2
0
*
Vehicle
MB
Sham
TBI
E)
IL-10 (Fold Change)
3
2
1
0
Vehicle
MB
Sham
TBI
F)
IL-4Rα (Fold Change)
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
MB
Sham
TBI

FIGURE 4

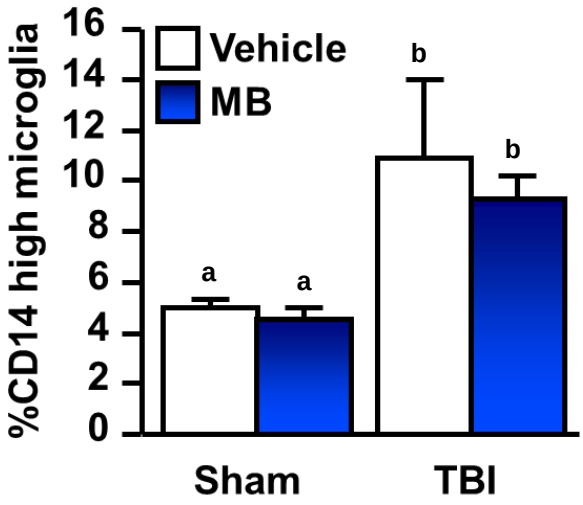
%CD14 high microglia
16
14
12
10
8
6
4
2
0
Vehicle
MB
a
a
b
b
Sham
TBI

FIGURE 5

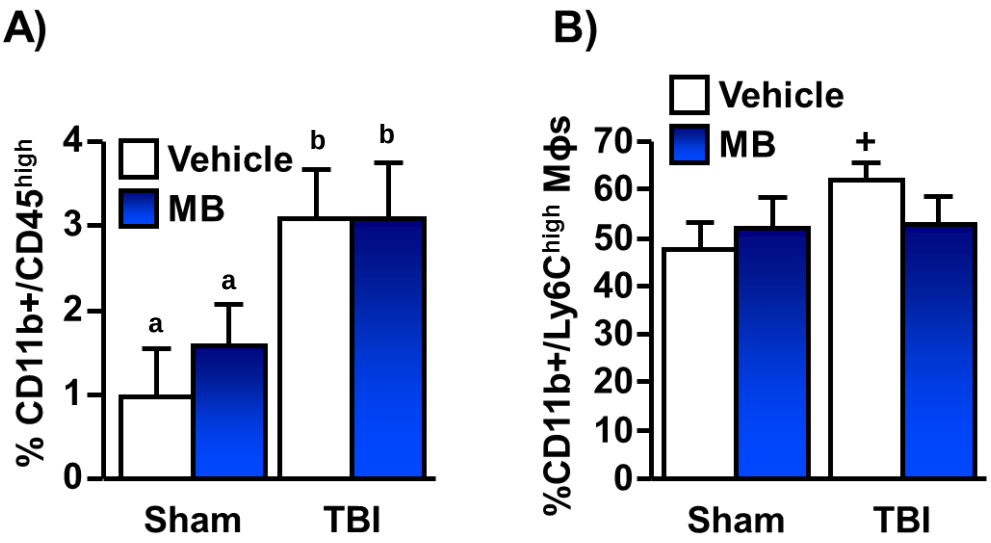
A)
% CD11b+/CD45high
Vehicle
MB
a
a
b
b
Sham
TBI
B)
%CD11b+/Ly6Chigh Mϕs
Vehicle
MB
+
Sham
TBI

FIGURE 6

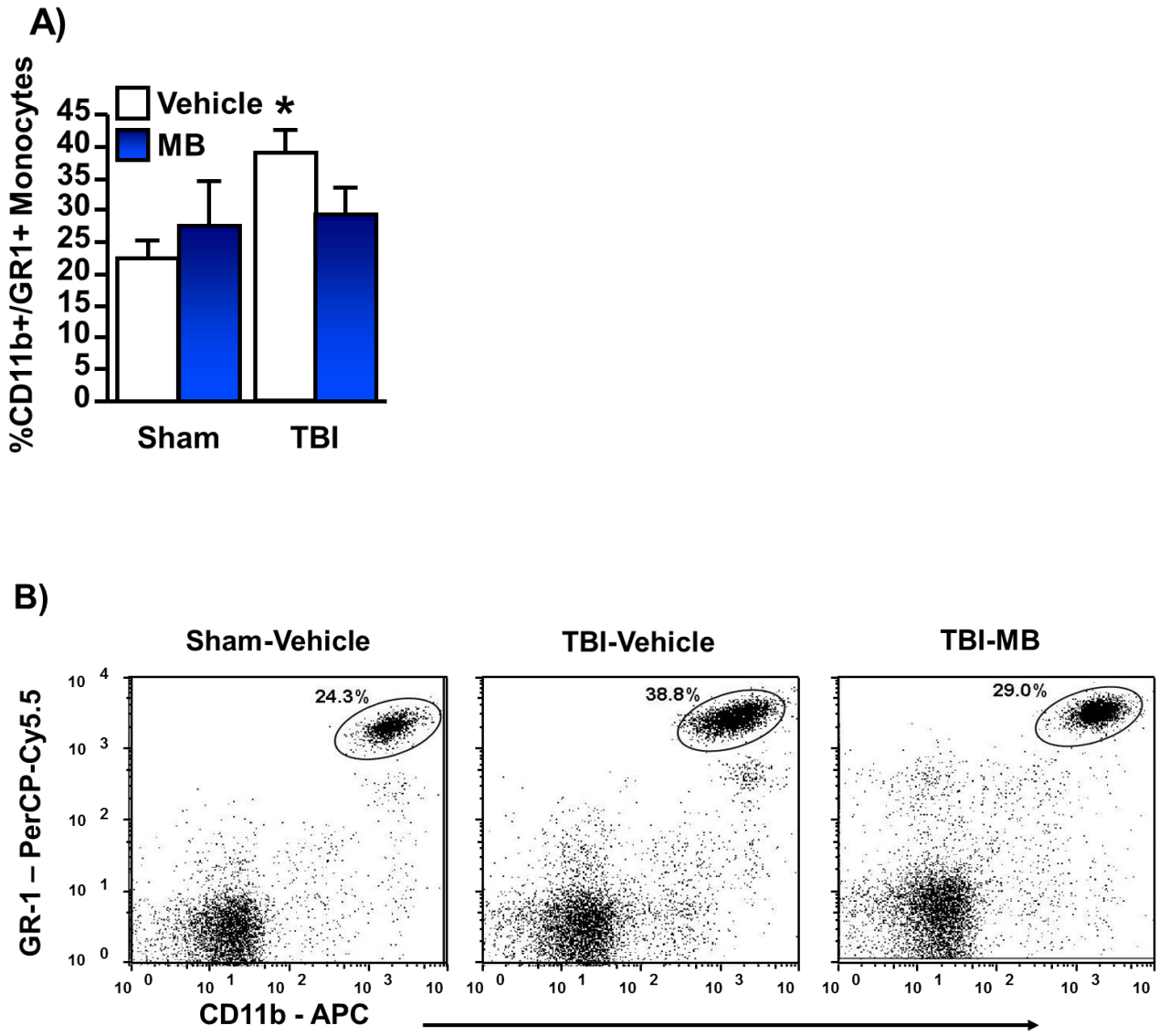
A)
%CD11b+/GR1+ Monocytes
Vehicle *
MB
45
40
35
30
25
20
15
10
5
0
Sham
TBI
B)
Sham-Vehicle
TBI-Vehicle
TBI-MB
GR-1 – PerCP-Cy5.5
CD11b - APC
24.3%
38.8%
29.0%

FIGURE 7

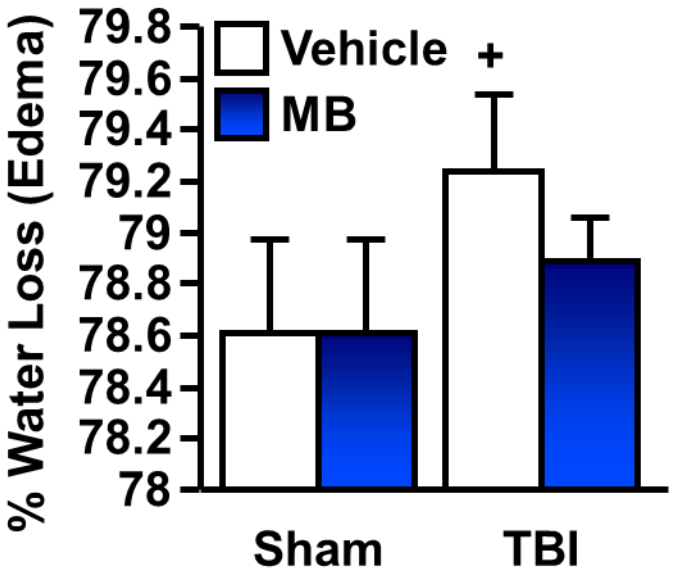
% Water Loss (Edema)
79.8
79.6
79.4
79.2
79
78.8
78.6
78.4
78.2
78
Vehicle
+
MB
Sham
TBI

FIGURE 8

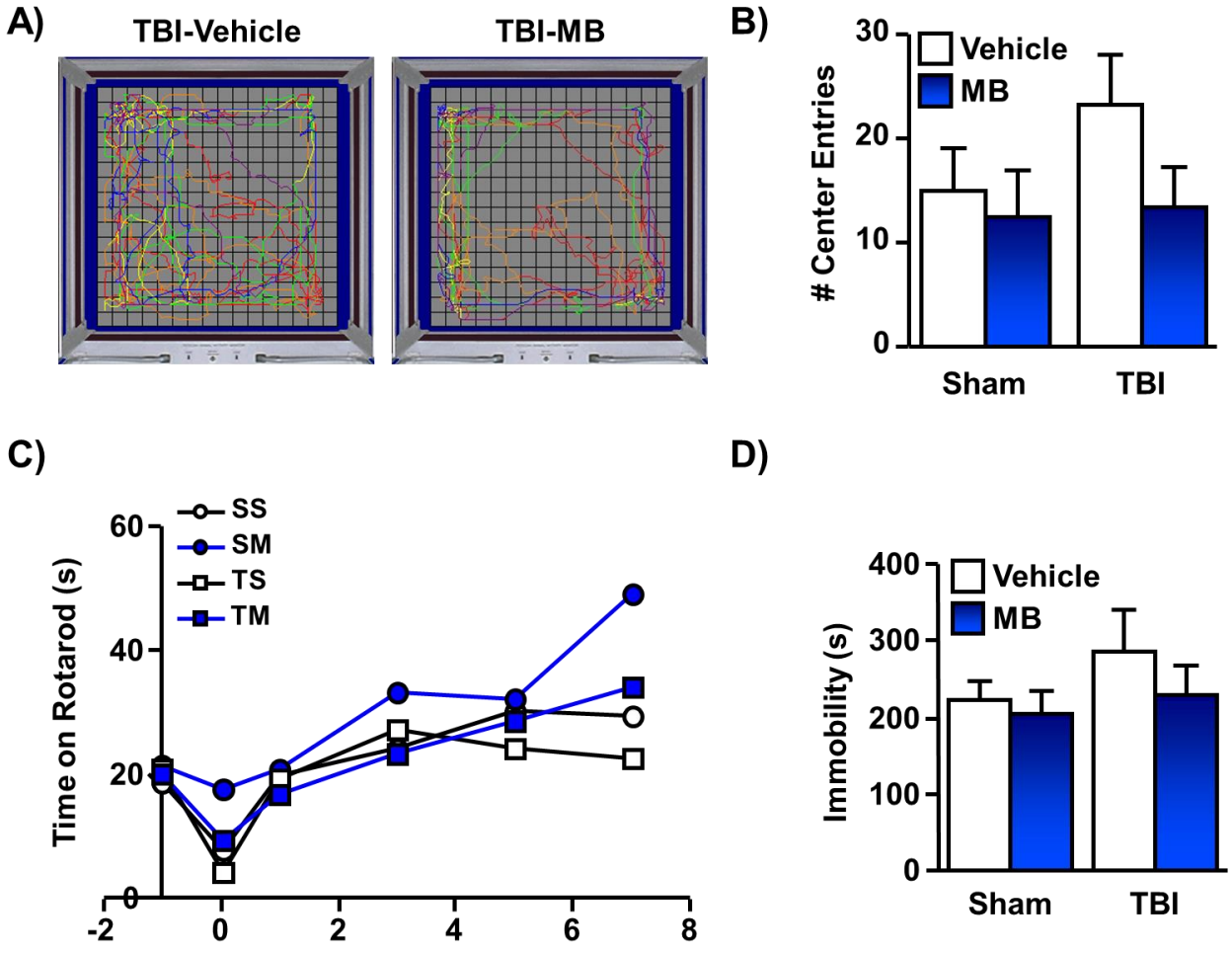
A)
TBI-Vehicle
TBI-MB
B)
30
20
10
0
Vehicle
MB
Center Entries
Sham
TBI
C)
SS
SM
TS
TM
60
40
20
0
Time on Rotarod (s)
-2
0
2
4
6
8
D)
400
300
200
100
0
Vehicle
MB
Immobility (s)
Sham
TBI

FIGURE LEGENDS

Fig. 1 Hypothetical Effects of MB Treatment on Secondary Injury in TBI. Adult mice (2-3 mo) received a moderate, diffuse TBI and were examined for neuroinflammation, edema formation, and behavioral deficits following injury. MB treatment was hypothesized to A) attenuate microglial activation (Fig. 3 and Fig. 4), B) ameliorate inflammatory cytokine upregulation (Fig. 2 and Fig. 3), C) prevent reactive oxygen and reactive nitrogen species formation, D) lessen blood-brain barrier disruption, E) inhibit macrophage trafficking (Fig. 5), F) reduce edema (Fig. 7), G) and abrogate behavioral deficits (Fig. 8) associated with TBI. *Reactive oxygen species (ROS), reactive nitrogen species (RNS), blood-brain barrier (BBB).*

Fig. 2 MB Abrogated TBI-induced Inflammatory Mediators in the Brain. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). After 24 h mice were sacrificed, the brain was collected, and a 1 mm coronal section was dissected. mRNA concentrations of A) IL-1β, B) iNOS, C) CD14, D) arginase, E) IL-10, F) IL-4Rα, and G) IL-4 were determined by qPCR. Bars represent the mean ± SEM. No significant interactions were identified.

Fig. 3 MB Abrogated TBI-Induced Expression of Inflammatory Mediators in Microglia. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). After 24 h a coronal brain section was removed for gross mRNA analysis (Fig. 2), and microglia were isolated from the remainder of the brain tissue. mRNA concentrations of A) IL-1β, B) CD14, C) iNOS, D) arginase, E) IL-10, and F) IL-4Rα in microglia were determined by qPCR. Bars represent the mean ± SEM. Arginase I was significantly upregulated following injury (TBI *x* Vehicle) compared to Sham *x* Vehicle and

TBI *x* MB (p = < 0.03) indicated by (*) and to a lesser extent Sham *x* MB (p = 0.10). No other significant interactions were identified.

Fig. 4 MB Did Not Reduced CD14 Protein Expression on Microglia after TBI. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). After 24 h microglia were isolated and CD14 protein expression was determined by flow cytometry. Quantitative analysis for $CD14^{high}$ expression is shown. Bars represent the mean ± SEM. CD14 expression significantly increased with TBI (p = 0.0004) indicated by (b) but there was no significant reduction following MB treatment (p = 0.6).

Fig. 5 MB Had No Effect on Trafficking Macrophages after TBI. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). Microglia/macrophages were isolated from the brain and analyzed by flow cytometry for the relative percentage of A) $CD11b^{+}/CD45^{high}$ and B) $CD11b^{+}/Ly6C^{high}$ cells. Bars represent the mean ± SEM. $CD45^{high}$ expression of $CD11b^{+}$ cells significantly increased with TBI (p = 0.015) indicated by (b) but there was no significant reduction with MB treatment (p = 0.65). In the analysis of $CD11b^{+}/Ly6C^{high}$ no significant differences were attributed to TBI (p = 0.18) or MB (p = 0.66); however, there was a minor interaction between the Sham *x* Vehicle and TBI *x* Vehicle groups (p = 0.08) that was also observed through *Post-hoc* analysis (p = 0.06) indicated by (+).

Fig. 6 MB Attenuated the Inflammatory Profile of Myeloid Cells in Circulation. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). After 24 h blood was analyzed for the percentage of $CD11b^{+/}GR\text{-}1^{+}$ cells in circulation by flow cytometry. Representative dot plots (A) and

quantitative analysis are shown (B). No significant interactions were identified; however, there was a minor interaction associated with TBI (p = 0.07) indicated by (*) that was reversed by MB (p = 0.14).

Fig. 7 MB Reduced TBI-induced Edema. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). The left and right cortices were dissected out after 24 h and were weighed. Cortices were oven-dried at 72 °C for 24 h and weighed to calculate percent water loss. Bars represent the mean ± SEM. No significant interactions were identified; however, the TBI *x* Vehicle group differed from the Sham *x* Vehicle group by a simple, one-tailed T-Test (p = 0.10) indicated by (+). No other interactions by a simple, one-tailed T-test were observed between the remaining groups.

Fig. 8 MB Reversed TBI-induced Deficits in Anxiety, Motor Coordination, and Depressive-like Behaviors. Adult (2-3 mo) mice received a sham injury or moderate TBI and were immediately injected (i.v.) with vehicle or 1 % methylene blue (2 mg/kg). Mice were tested for anxiety behavior in an open field setup. Representative travel plots are shown in (A) and the number of entries into the center in (B). Furthermore, mice were tested for motor coordination on Rotarod from -2 to 8 d post-TBI (C). At 7 d following injury, mice were tested for depressive-like behavior using a tail suspension test. Immobility times are shown in (D). Bars represent the mean ± SEM. No significant interactions were identified.

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